

FIGURE 1

POTENTIAL ENHANCEMENT OF LONG-TERM STABILITY OF RTCC

Sample	Growth medium	7/21/2001	7/24/2001	8/15/2001	8/16/2001	8/16/2001	8/20/2001
1	SOB/Mg ⁺⁺ only	< 10 ⁵ (0)	0	1 X 10 ⁴ (1)	1 X 10 ⁴ (1)	1 X 10 ⁴ (1)	1 X 10 ⁴ (1)
2	1 + 100mM NaCl	1.5 x 10 ⁶	2.6 X 10 ⁵	1.87 X 10 ⁶	1.78 X 10 ⁶	1.29 X 10 ⁶	1.7 X 10 ⁶
3	1 + 200mM NaCl	2.37 x 10 ⁶	8.7 X 10 ⁵	5.8 X 10 ⁶	7.51 X 10 ⁶	2.04 X 10 ⁶	9.5 X 10 ⁶
4	1 + 300mM NaCl	3.9 x 10 ⁶	7.2 X 10 ⁵	1.73 X 10 ⁶	4.27 X 10 ⁶	1.32 X 10 ⁶	1.04 X 10 ⁶
HS TIME		45 SECS	60 SECS	60 SECS	60 SECS	60 SECS	60 SECS
VIAL SIZE		SMALL	SMALL	SMALL	SMALL	LARGE	Lg w/beads

Variations in dessication medium, competence medium, and growth medium : RTCC

#	GROWTH IN	COMPETENCE MEDIA	DESSICATION	TE (day3) x 10 ⁶ cfu/ug Avg of 3	Survival x10 ⁷ cfu/ml	TE(d62) x10 ⁶ upt w/o S	Survival x10 ⁷ cfu/ml
1	SOB.Mg	FSB	20%TreSorb*	0.9	9	0.17	0.1
2	SOB.Mg.NaCl	FSB+20%Fructose	20%TreSorb*	3	208	4.3	25
3	SOB.Mg.NaCl	FSB+20%Fructose	TGGS**	1.3	330	42	72
4	SOB.Mg.NaCl	FSB+20%Fructose	TGMS***	5.7	249	24	65
5	SOB.Mg.NaCl	FSB+10%MAG	20%TreSorb*	1.33	122	1.4	15
6	SOB.Mg.NaCl	FSB+10%MAG	TGGS**	6.5	182	13	40
7	SOB.Mg.NaCl	FSB+10%Sorb	20%TreSorb*	3.44	151	4.4	36.6
8	SOB.Mg.NaCl	FSB+10%Sorb	TGGS**	9.7	239	23	65.8
9	SOB.Mg.NaCl.Casein.Maltose	FSB+20%Fructose	20%TreSorb*	1.8	101	2.9	~35
10	SOB.Mg.NaCl.Casein.Maltose	FSB+20%Fructose	TGGS**	6.73	180	11.4	50.8
11	SOB.Mg.NaCl.Casein.Maltose	FSB+20%Fructose	TGMS***	6.61	201	19.5	28.4
12	SOB.Mg.NaCl.Casein.Maltose	FSB+10%MAG	20%TreSorb*	0.91	110	1.5	~17.5
13	SOB.Mg.NaCl.Casein.Maltose	FSB+10%MAG	TGGS**	5.4	270	16	~30
14	SOB.Mg.NaCl.Casein.Maltose	FSB+10%Sorb	20%TreSorb	1.26	83	4.62	~20
15	SOB.Mg.NaCl.Casein.Maltose	FSB+10%Sorb	TGGS**	8.03	279	48.2	~80
16	SOB.Mg.NaCl.Casein.Maltose	FSB+10%Sorb	TGMS***	12.3	218	22	~70

* 20%Tresorb = A solution of 20%trehalose (Sigma) in water, to which a final concentration of 2.5%Sorbitol has been added

**TGGS = A solution of Trehalose, Glutamic acid, Gelatin and 2.5% Sorbitol

***TGMS + A solution of Trehalose, Gelatin, Alpha-methyl glucopyranoside and 2.5% Sorbitol.

Day 62 : Rehydration was in upt buffer w/o sucrose.

FIGURE 2

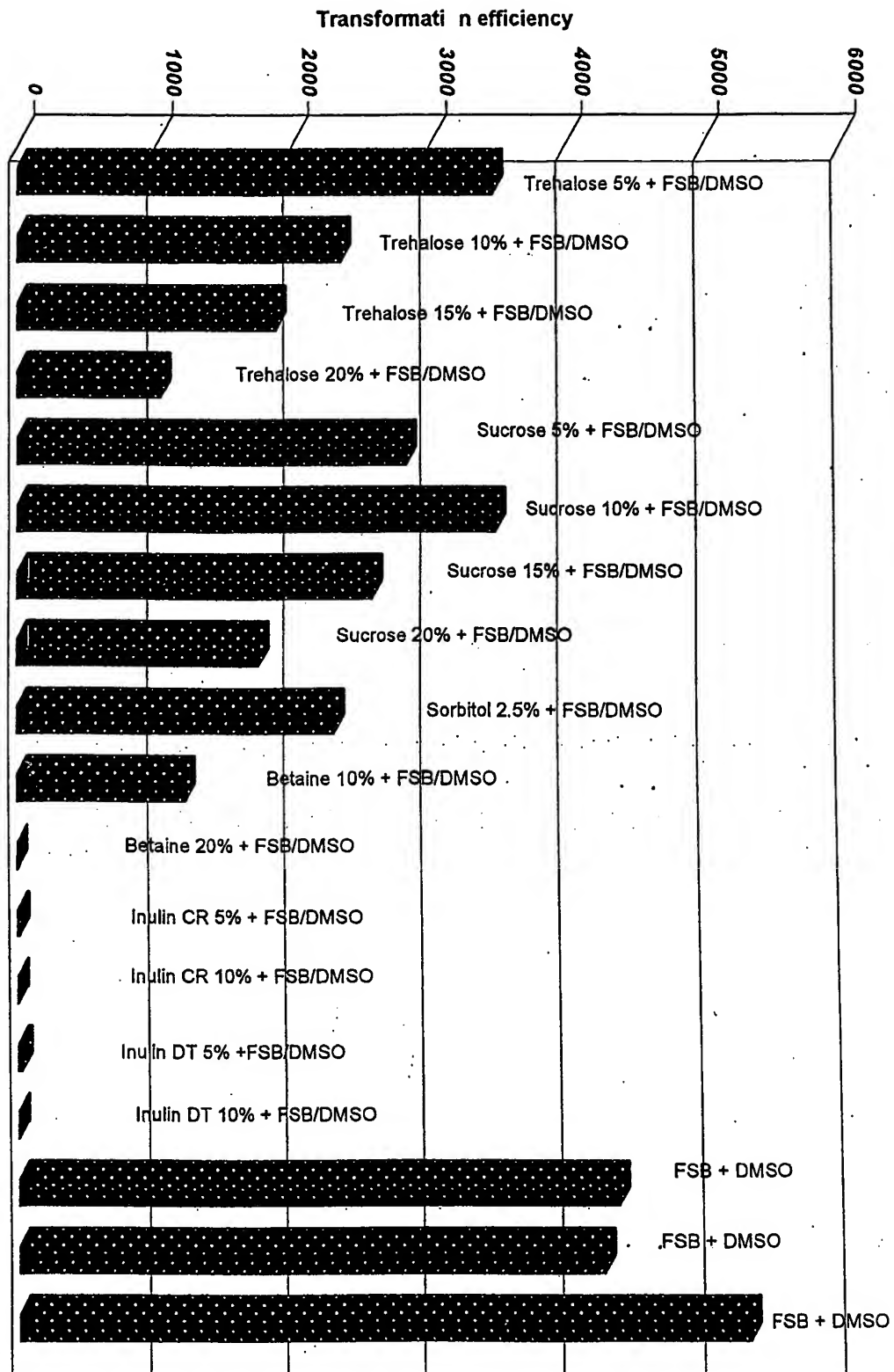
EFFECT OF VARIATIONS IN GROWTH MEDIUM, COMPETENCE MEDIUM, AND DESSICATION MEDIUM ON T.E. OF RTCC

Sample	Growth Medium	Competence Medium	Dessication Medium	Avg. cfu/ug pUC *	Incr. In TE over std. conds.
1	SOB-Mg	FSB	20% Trehalose	2×10^4	
2	SOB-Mg	FSB	20% Trehalose	3×10^4	
3	SOB-Mg	FSB	Tre-Suc	1×10^5	4x
4	SOB-Mg	FSB + 10% FRUCTOSE	Tre-Suc	2.3×10^5	9.2x
5	SOB-Mg	FSB + 20%FRUCTOSE	Tre-Suc	5.7×10^4	2.3x
6	SOB-Mg	FSB + 20%FRUCTOSE	Tre-Suc + BSA [§]	2.7×10^4	
7	SOB-Mg-200mM NaCl	FSB	20% Trehalose	1.56×10^6	62.4x
8	SOB-Mg-200mM NaCl	FSB + 10% FRUCTOSE	Tre-Suc	3.19×10^6	127.6x
9	SOB-Mg-200mM NaCl	FSB 20%FRUCTOSE	Tre-Suc	5.41×10^6	216.4x
10	SOB-Mg-200mM NaCl	FSB + 20%FRUCTOSE	100ul 20%Fructose, 40min → Tre-Suc	4.09×10^6	163.6x
11	SOB-Mg-200mM NaCl	FSB + 20%FRUCTOSE	Tre-Suc + BSA [§]	4.41×10^6	176.4x
12	SOB-Mg-200mM NaCl	FSB + 20%FRUCTOSE	Tre - Suc + + Pro/Gly [§]	5.11×10^6	204x

[§] Concentration of BSA, Proline, or Glycine was 100ug/ml of the Trehalose, or Trehalose+Sucrose mixture

FIGURE 3

FIGURE 4



OPTIMIZATION OF CONDITIONS FOR GENERATING CHEMICOMPETENT RTCC

#	GROWTH IN	COMPETENCE MEDIA	PREINC. 50u, 10min	DESSICATION	T.E., Day 2 (x10 ⁶)				T.E., Day 20 (x10 ⁶)				Surv.(day20) cfu/ul X 10 ⁴	TE(day 60) x 10 ³	TE (Day 92) X 10 ³ (FSB)	Surv(day92) cfu/ul x10 ⁴	TE(day~180) x 10 ³ (uptw/o)
					FSB	UPT	MIX	FSB	UPT	MIX	FSB	UPT					
A1	SOB.Mg.NaCl	FSB		10%TreSorb*	1.98	9.94	6.9	1.11	8.1	5.05	1.11	8.1	200	0.8	1.69	~650	4.28
A2	SOB.Mg.NaCl	FSB		20%TreSorb	2.9	6.92	6.05	2.3	5.1	0.33	2.3	5.1	54	0.46	0.27	~325	3.61
A3	SOB.Mg.NaCl	FSB+20%Fructose		10%TreSorb*	4.4	4.67	4.95	0.63	10.33	0.28	0.63	10.33	120	1.51	0.73	73	0.59
A4	SOB.Mg.NaCl	FSB+20%Fructose		20%TreSorb	1.86	13.2	3.95	1.05	16.35	0.39	1.05	16.35	450	5.72	2.86	~2000	7.05
A5	SOB.Mg.NaCl	FSB+20%Fructose		8:1:1 TSGG**	9.18	11.9	15	0.96	8.15	0.79	0.96	8.15	143	2.68	1.26	~800	5.07
A6	SOB.Mg.NaCl	FSB+20%Fructose		8:1:1 TSGG+Sorb	6.18	17.06	14.15	1.92	17	1.44	1.92	17	360	2.24	3.02	~2500	11.08
A7	SOB.Mg.NaCl	FSB+20%Fructose	15%Fru	8:1:1 TSGG+Sorb	10.82	32.42	24.23	1.37	17.08	0.69	1.37	17.08	400	2.49	1.36	~2200	SNA
A8	SOB.Mg.NaCl	FSB+20%Fru+Pro+Threo		8:1:1 TSGG+Sorb	3.26	19.13	17.37	2	13.8	6	2	13.8	400	2.07	1.53	~1200	11.02
A9	SOB.Mg.NaCl	FSB+20%Fru+Pro+Threo	15%Fru	8:1:1 TSGG+Sorb	5.84	7.06	10.84	1.31	9.37	2.78	1.31	9.37	253	1.84	2.12	~3000	5.47
A10	SOB.Mg.NaCl	FSB+20%Fru+Pro+Threo		10%TreSorb*	3.73	9.35	8.23	0.44	14.71	0.9	0.44	14.71	188	2.38	0.9	~800	3.98
B1	SOB.Mg.NaCl.Casein	FSB		20%TreSorb	2.34	3.53	3.62	0.68	3.59	0.49	0.68	3.59	71	0.53	0.44	~400	SNA
B2	SOB.Mg.NaCl.Casein	FSB+20%Fructose		20%TreSorb	7.02	7.7	11	1.91	20.81	0.99	1.91	20.81	300	1.97	3.86	~5000	15.69
B3	SOB.Mg.NaCl.Casein	FSB+20%Fructose		8:1:1 TSGG	1.42	4.3	2.42	0.12	1.75	0.25	0.12	1.75	92	0.82	1.08	~1100	SNA
B4	SOB.Mg.NaCl.Casein	FSB+20%Fructose		8:1:1 TSGG+Sorb	3.88	4.1	8	3.5	13.29	3.12	3.5	13.29	300	3.2	2.39	~4000	10.56
B5	SOB.Mg.NaCl.Casein	FSB+20%Fructose	15%Fru	8:1:1 TSGG+Sorb	4.17	3.14	6.84	1.32	1.22	0.39	1.32	1.22	250	1.04	0.06	67	
B6	SOB.Mg.NaCl.Casein	FSB+20%Fru+Pro+Threo		8:1:1 TSGG+Sorb	3.04	4.63	16.65	2.25	32.77	11.38	2.25	32.77	115	2.57	0.19	96	7.82
B7	SOB.Mg.NaCl.Casein	FSB+20%Fru+Pro+Threo	15%Fru	8:1:1 TSGG+Sorb	3.41	0.53	5.77	0.38	0.7	0.33	0.38	0.7	23	1.05	0.16	197	1.78
B8	SOB.Mg.NaCl.Casein	FSB+20%Fru+Pro+Threo	15%Fru	20%TreSorb	1.82	3.26	5.81	0.66	4.94	1.96	0.66	4.94	320	0.98	1.75	~1500	3.67
B9	SOB.Mg.NaCl.Casein	FSB+20%Fructose		10%TreSorb*	7.42	2.17	11	1.22	0.93	0.37	1.22	0.93	150	0.56	0.39	163	0.3
B10	SOB.Mg.NaCl.Casein	FSB+20%Fructose	15%Fru	10%TreSorb*	3.85	3.49	9.91	0.73	5.85	0.73	0.73	5.85	110	0.87	0.6	259	0.46
C1	SOB.Mg	FSB		10%TreSorb*	5.37	1.97	14.94	0.96	2.24	0.65	0.96	2.24	100	0.44	0.34	112	0.42
C2	SOB.Mg	FSB		20%TreSorb	1.2	0.84	1.79	0.43	0.1	0.12	0.43	0.1	60	0.2	0.26	105	0.32
C3	SOB.Mg	FSB+20%Fructose		8:1:1 TSGG+Sorb	1.2	0.79	0.8	0.05	0.35	0.04	0.05	0.35	4	0.06	0.03	16	0
C4	SOB.Mg	FSB+20%Fructose		10%TreSorb*	1.2	0.05	0.06	0.03	0.4	0.01	0.03	0.4	15	0.12	0.04	~26	0

SNA = Sample Not Available

SNA = Sample Not Available
upt w/o = upt buffer without sucrose

FIGURE 5

Process Stage	Improvement/ Addition	Effect of addition/improvement	Approx. Improvement in XL10Gold Dessicated cells (comp. to control)
GROWTH	Addition of NaCl(200mM) to growth medium, before seeding culture	Induction of osmotic stress, resulting in enhanced synthesis of osmoprotective sugars (internal trehalose concentration increased)	At least 10-fold, but more importantly, extends RT shelf life from only few weeks to > 5 months.
	Addition of hydrolyzed Casein extract to growth medium	Reduces the effect of proteolytic enzymes within the cell, enhancing dessication survival and rehydration survival	~3 to 5-fold, depending on the dessication medium
	Addition of Maltose (0.15 - 3% final) to the growth medium	Reduces use of internal trehalose as metabolite, increases trehalose availability for osmoprotection	increase (by ~2-fold) the shelf-life of product at RT
	Harvesting at mid log phase (~0.45 to ~0.5 OD ₅₅₀)	Improves tolerance to the stress of dessication	~2- fold
	20% Fructose in FSB w/o the glycerol	Enhances long-term survival, and reduces the inhibitory effect of trehalose on T.E.	>4-fold, depending on dessication / growth medium. Long term~30-fold
	Addition of Proline and Threonine in competence medium	Appears to enhance survival during dessication	~4-fold, depending on growth medium
PRE-INCUBATION	Pre-incubation of competent cells in 10-25% Fructose prior to addition of dessication medium	Enhances both dessication survival, TE, and long-term RT stability	Up to ~ 10-fold, depending on other processing conditions
	Reduction in concentration of trehalose to ~8%-15% final	Improves TE, and survival	
DESSICATION MEDIUM	Inclusion of Sucrose (~2-8% final)	Improves TE, and survival, produces lighter "foam"	
	Inclusion of Gelatin (Prionex)(0.5-2%)	Provides bulking, excellent "foam", better TE	2-10 fold, varies with dessic. medium
	Inclusion of Sorbitol (final 1-3%)	Reduces TE slightly, but greatly improves survival	
	Glutamate (monosodium salt)	Enhances dessication resistance, and shelf-life	
	Use of amber glass vials for samples	Greatly improves long -term stability at RT due to reduction in light-mediated "aging" of dried cells	
	Oxygen scrubbers during the process of dessication	Reduces deleterious effect of oxygen during the most stressful period of dessication	
DESSICATION CONDITIONS	Vacuum packing	Reduces the effect of oxygen in the packaged product, thereby allowing true stability	
	Baking of rubber stoppers	Greatly improves long -term stability at RT due to reduction in moisture reabsorption from the stoppers	
	ZnCl ₂ , SnCl ₂ in Rehydration Soln.	Stabilization of proteins during the period of rehydration,	~2-fold to 3-fold
REHYDRATION	RbCl ₂ , Hex. CoCl ₂ , CaCl ₂ , MnCl ₂	Encourage competence induction	

FIGURE 6